Transcriptional Regulators of the 51D Surface Protein Gene of Paramecium tetraurelia

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Received May 15, 2002; Accepted August 6, 2002

The α-51D gene encodes surface protein 51D, which specifies serotype 51D of Paramecium tetraurelia stock 51. Previously, we isolated the gene as one expressed at much higher levels in a short-lived mutant than in its parental wild-type stock and characterized its expression to be up-regulated with increased clonal age of the wild-type stock. Here we investigated the transcriptional mechanism for the serotype expression in light of its possible causal relationship to the Paramecium clonal life span. DNA-protein binding analyses of the upstream of the α-51D gene identified a stretch of DNA sequence that interacted specifically with macronuclear proteins prepared from the 51D-expressing mutant. The DNA sequence was mapped to the 23 bp between -310 and -288, counting from the initiation position of 51D transcription. A protein with a binding ability for this DNA element was purified to homogeneity from the macronuclear proteins by chromatography using the specific DNA-protein interaction. In vitro transcriptional analyses showed that both the purified protein and its target domain are essential for increased synthesis of the 51D transcript. These results showed that the DNA-protein interaction is required for induction of the 51D expression in the mutant. However, aged wild-type paramecia, in which the serotype is also highly expressed, lacked the same specific transcriptional activity as detected in the mutant, suggesting another DNA-protein interaction involved in the 51D up-regulation.

Key words: life span; aging; surface protein 51D; Paramecium tetraurelia

The outer surface of Paramecium cells is covered with one of a family of membrane proteins of unknown function. The proteins have been known as immobilization antigens or surface proteins, and paramecia with specific surface proteins are called serotypes.1,2 The cells have a range of easily distinguishable, alternative types, which are generally mutually exclusive and can be made to change reversibly. In the best studied strain, Paramecium stock 51, there are 14 different serotypes, designated 51A, 51B, 51C, etc., and probably others that have not yet been identified. Among them, the 51D serotype is strikingly different from other types in that the 51D gene is a multigene family of the D type isogenes, in which the α-51D gene is exclusively transcribed in cells expressing the serotype.3

For the final purpose of identifying the causal determinant of the Paramecium clonal life span, the jumyo gene,4,5 we examined differences in gene expression between wild-type stock 51 and its mutant d4-SL4 with a short clonal life span, and isolated three genes that showed expression patterns dependent on clonal age.5,6 Structural and transcriptional analyses showed that two of the three were genes for serotype, 51A and α-51D, of which the former was gradually repressed as clonal aging proceeded while the latter was reciprocally activated with aging.

These serotype genes, however, are probably not our final target. Instead, we suppose that the gene product of jumyo serves as the nuclear factor that specifies the clonal life span by ultimately governing essential cellular functions accompanied with age-dependent phenomenon including the above serotype switching. If this holds true, understanding the mechanism for serotype expression would give some clues for isolation of the jumyo gene. Since expression of Paramecium genes is considered to be usually regulated at the stage of transcription,6,9 our next interest was directed toward the underlying molecular process for transcriptional regulation of the two serotype genes. Here we dealt with the transcriptional mechanism for the α-51D gene.

Materials and Methods

Culture of paramecia. P. tetraurelia wild-type stock 51 and the jumyo mutant d4-SL4 were used and cultivated as done previously.5,8

Preparation of macronuclear proteins. An age-synchronized cell population of young stock 51 (about 15 fissions old) and aged stock 51 (about 150 fissions old) as well as the jumyo mutant were used as sources of protein. Cells were harvested from a culture of 400–500 cells/ml and subjected to cellular fractionation for isolation of macronuclei.9

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proteins were extracted from the macronuclear fraction with the NE-PER nuclear extraction reagent (PIERCE) and dialyzed overnight against the undermentioned binding buffer using a dialysis membrane with a molecular mass cut-off of 5,000. The dialyzed samples were used as the macronuclear extract in the following experiments.

**Gel retardation assay.** Probe DNAs longer than 100 bp were generated by PCR. Primers were designed on the basis of the nucleotide sequence of the α-51D genomic DNA (accession No.: X96400) and the genomic DNA as the template for PCR was prepared by a phenol method. Shorter probe DNAs were chemically synthesized. These probe DNAs were labeled with biotin using the biotin labeling kit (PIERCE).

The DNA-binding experiment was done nonisotopically as follows: Protein (10 μg in the macronuclear extract or 10 ng of purified preparation) was incubated with 400 ng of denatured salmon sperm DNA (0.5–1 kb) and, when necessary, 100 ng of unlabeled probe DNA as a competitor in 19 μl of the binding buffer (10 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 8 mM MgCl₂, 1 mM dithiothreitol and 10% glycerol) for 20 min on ice, and then 1 μl of biotin-labeled probe DNA (1 ng) was mixed with the reaction mixture. After an additional 30 min, the mixture was put on a 6% polyacrylamide gel ((8 cm × 8 cm × 1 mm) containing 0.25 X TBE (22.5 mM Tris, 22.5 mM boric acid, and 1 mM EDTA) and electrophoresed at 100 V until bromophenol blue reached the bottom of the gel. The labeled probe DNA was then electroblotted to a nylon membrane in 0.25 X TBE at 4 mA cm⁻² for 30 min and detected using the LightShift chemiluminescence kit (PIERCE) and an ECL Mini-camera (Amersham).

**DNA affinity chromatography.** The principle of this procedure is based on a specific interaction between biotinylated ligand DNA immobilized on streptavidine-conjugated agarose beads (PIERCE) and proteins recognizing the nucleotide sequence of the ligand DNA. After immobilization of the ligand, the streptavidine gel, 0.5 ml, was packed into a column and equilibrated with 2.5 ml of the above binding buffer. The macronuclear extract, 50 μg in protein, was incubated with 2 mg of denatured salmon sperm DNA (0.5–1 kb) for 20 min on ice and put onto the column. Following a thorough washing of the column with 2.5 ml of the binding buffer containing 0.2 M NaCl, proteins bound to the ligand DNA were eluted with 1.5 ml of the binding buffer containing 0.5 M NaCl. The purified proteins were dialyzed against the binding buffer as described above for use in the subsequent experiments.

**In vitro run-off transcription.** Two μl of the macronuclear extract was added singly or in combination with 10 ng of purified protein to 18 μl of the binding buffer containing 100 ng of template DNA, 1 mM each of ATP, CTP, and GTP, 650 μM of UTP, 350 μM of biotin16-UTP and 40 units of the ribonuclease inhibitor RNasin. Transcription was done at 25°C for 1 h. Electrophoresis of the transcript (2 μl) and the subsequent transfer to nylon membranes followed our protocol for Northern blotting. Signals from biotinylated transcripts were detected as described under "Gel retardation assay".

**Other experiments for protein.** Protein concentration was measured by the method of Bradford with bovine γ-globulin as the standard protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done following Laemmli. Gels after the run were stained with a fluorescent dye.

**Results and Discussion**

**DNA-protein interaction on the upstream of the α-51D gene**

In our preceding study, the α-51D gene was found to be transcribed at background level when wild-type paramecia were young, but its transcription was prominent in aged wild type as well as the short-lived jumyo mutant. This differential gene expression is supposed to result from transcriptional regulation taking place upstream of the serotype gene: there should exist a trans-acting nuclear protein factor(s) that responds to a cis-active DNA element(s) located anywhere on the upstream regulatory region, by which the gene expression is up- or down-regulated.

Assuming that such an intermolecular regulatory interaction occurs preferentially in young wild type or the jumyo mutant, we searched the macronuclear proteins of the two stocks for DNA-binding activities specific to the serotype gene by a gel retardation assay using ten kinds of DNA fragments covering the upstream nontranscribed region as the probe (Fig. 1). First, two fragments, Probes 1 and 2, spanning –571 to –23 (just 5’ to the putative promoter TATAAA) relative to the start of the transcription were examined for interaction with proteins in the macronuclear extract. In the assay using the proteins of the mutant in which the 51D serotype is actively expressed, the more upstream DNA fragment, Probe 1, which precedes position –288, caused an upward band shift indicating the formation of a protein-DNA complex, but the mobility of Probe 2 was not affected with the same protein preparation (data not shown). On the other hand, proteins of young wild type expressing little 51D serotype did not show such an affinity for Probe 1 as well as for Probe 2 (not shown). Thus, we detected a DNA-binding activity toward the 51D upstream region only in macro-
nuclear proteins of the *jumyo* mutant and thereafter exclusively investigated the mutant macronuclear extract.

We next divided the Probe 1 region into three parts (Probes 3 to 5 in Fig. 1) and assayed them for response to the mutant macronuclear proteins. In this screening, the protein sample was found to preferentially retard the mobility of Probe 5, located between positions −383 and −288 (data not shown). We did further mapping by subdividing the DNA sequence of Probe 5 into three (Probes 6 to 8 in Fig. 1) and finally narrowed down the target sequence to Probe 8, the 23 bp present ahead of position −288 (lane 2 in Fig. 2). The shifted DNA band disappeared when an excess of unlabeled Probe 8 was added as a competitor in the reaction mixture (lane 3), which demonstrated that the DNA-protein interaction is sequence-specific.

A close examination of the 23-bp domain detected one each of a direct repeat and an inverted repeat of the nucleotide sequence, both of which are comprised of the consensus sequence 5′-A(T/G)TGTGTT-3′ (Fig. 1). Since many transcriptional regulator proteins function through binding to repeated DNA sequences in their target regions, either or both of the repeats of the octameric sequence is likely to participate in the direct contact with the macronuclear proteins. To distinguish between these possibilities, deletions of Probe 8 were assayed for retaining the affinity with the proteins. A partial deletion in the 5′-half of both repeats made the resultant DNA fragment, Probe 9 (Fig. 1), inactive in the binding assay (lane 5 in Fig. 2). In contrast, Probe 10, which carries the 3-bp deletion in the 3′-half of the inverted repeat with the direct repeat intact (Fig. 1), presented a band shift pattern similar to that of Probe 8 (compare lanes 2 and 7 in Fig. 2). These observations seem to favor identifying only the direct repeat as the target sequence. Still, the somewhat weakened intensity of the shifted band seen for Probe 10 in comparison to that of Probe 8 suggests that the 3′-deletion may result in lowered binding efficiencies, which makes it difficult to rule out the involvement of the inverted repeat. A DNA database search did not find the octameric sequence in any transcriptional regulatory element which has so far been reported.

**Purification of a DNA-binding protein specific to the α-51D gene**

We attempted purification of proteins with the binding ability for the DNA sequence of Probe 8 by affinity chromatography using the probe DNA as the ligand. Macronuclear proteins of the mutant stock were separated by chromatography and proteins retained in the affinity column were eluted with the running buffer with increased salt concentration.

The profile of the eluted proteins was made visible by SDS-PAGE (Fig. 3): the track in which the proteins were run had one major protein band and a few faint ones (lane 3). To differentiate between all the eluted proteins participating in the specific contact with the ligand DNA or the eluted fraction being contaminated with non-specifically bound proteins, this fraction was put through the second round of chromatography and the new eluate was electrophoretically inspected for purity: the faint multiple band seen after the first purification was lost, while the major band of a protein with molecular weight of 26,000 appeared again with an unaltering intensity.
Proteins were electrophoretically size-fractionated and stained by UV fluorescence. Lanes: 1, molecular mass markers; 2, macronuclear extract prepared from the \textit{jumyo} mutant; 3, purified preparation after the first chromatography; 4, purified preparation after the second chromatography. The molecular masses of the marker proteins in lane 1 are given to the left of the lane.

Consequently, we ascribed the DNA-binding activity detected in the binding assay to this protein obtained after the repeated purification. In fact, the second purified preparation proved to retard the electrophoretic mobility of Probe 8 in DNA-binding assays as observed for the mutant macronuclear extract (data not shown), verifying this assignment.

A titration experiment showed that about 2 ng of the purified protein is equivalent in the ability to retard DNA migration to 10 \( \mu \)g of the mutant macronuclear proteins, indicating that about five thousandfold purification on the basis of DNA-binding activity was attained through the chromatography.

Transcriptional activation of the \( \alpha \)-51D gene

The molecular interaction between the protein thus purified and its 23-bp target DNA was functionally characterized by \textit{in vitro} transcription reactions on the \( \alpha \)-51D gene (the top panel in Fig. 4). As a positive control, the mutant macronuclear extract was incubated alone with a DNA fragment of the \( \alpha \)-51D gene beginning 320 bp ahead of and ending 918 bp past the transcriptional start site (−320 to +918) which contains the 23-bp sequence of interest (see Fig. 1), after which synthesis of RNA was begun. The transcript was run on a denaturing gel, transferred to a membrane, and detected as a chemiluminescent signal. Successful RNA synthesis would generate a transcript of about 900 bp long. Imaging of the signal gave a single band at a position corresponding to the expected size (lane 1), confirming that the nuclear extract can elongate nascent RNA chains while incorporating ribonucleoside-triphosphates including a biotin-labeled one. When the affinity-purified protein was added to the reaction, the transcription was found to be stimulated several times (lane 2). Since the input of the purified protein, 10 ng, is about five times the equivalent of the nuclear extract used here in terms of DNA-binding ability (see above), these two data together are represented as the synthesis of the 51D transcript in proportion to the DNA-binding activity, which is best explained by assigning the transcriptional increase to the binding of the protein to its target element. Actually, in the transcription experiments using a template DNA devoid of the 23-bp domain (the region −284 to +918), the relative intensity of signals from the resulting transcripts was below detectable levels with (lane 4) or without (lane 3) the protein.

Taken together, we concluded that the protein purified in this study serves as a \textit{trans}-acting factor for positive regulation of the 51D transcription through the specific interaction with the −310 to −288 domain, and thereby induces the 51D expression in the \textit{jumyo} mutant. Although the molecular basis for expression of \textit{Paramecium} serotype genes has been clarified to some extent for 51A\textsuperscript{18-20} which has been identified as another serotype showing an aging-dependent expression pattern,\textsuperscript{5} this paper is the first report concerning the 51D gene and so far no relevance in the underlying events for gene expression has been observed between the two serotypes.

Implication for molecular differences in the 51D transcriptional mode

The coincidental activation of the 51D expression
in the jumyo mutant and aged wild-type stock\(^3\) makes it plausible that the above transcriptional explanation is the case also for aged paramecia. Based on the analogy, we analyzed the macronuclear extract prepared from the aged cells for targeting the 51D upstream domain responsive to the mutant protein by a gel retardation assay (Fig. 2). Unexpectedly, however, the wild-type sample did not respond to any of the target DNA fragments: Probe 8 (lane 1), Probe 9 (lane 4), or Probe 10 (lane 6). The same results were obtained when probing with more extensive DNA regions (Fig. 1), Probe 1 and Probe 2 (data not shown).

Next, the in vitro transcription experiment to examine the 51D expression was done for the wild-type stock as it was done for the mutant (Fig. 4). The macronuclear extract from young wild type (the middle panel) which is used as a reference caused no detectable transcription on the DNA template accommodating the 23-bp element (lane 1) in agreement with our previous data showing little 51D expression in young paramecia.\(^4\) When combined with the mutant trans-activator, the transcription was induced to a level comparable to that observed for the mutant macronuclear extract (lane 2). The stimulation effect did not appear in the absence of the responsive element (compare lines 3 and 4). This experiment indicates that the same basal transcription machinery includ-

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**References**


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